Detection and phylogeny of *Wolbachia* in field-collected *Aedes albopictus* and *Aedes aegypti* from Manila City, Philippines

Maria Angenica F. Regilme[1,2], Tatsuya Inukai[1], and Kozo Watanabe[1,2]

[1] Center for Marine Environmental Studies (CMES), Ehime University, Matsuyama, Ehime, Japan

[2] Ehime University - De La Salle University International Collaborative Research Laboratory, Laguna Province, Philippines

Corresponding author

E-mail: watanabe.kozo.mj@ehime-u.ac.jp

Abstract

Wolbachia is the most common bacterial endosymbiont of arthropods, such as the medically important *Aedes albopictus* and *Aedes aegypti.* Both *Wolbachia* and mitochondrial DNA are maternally inherited. This study collected 12 adult *Ae. albopictus* and 359 *Ae. aegypti* from 183 households in a dengue-prone area, Manila, Philippines, between June and September 2017. *Aedes* larvae (n = 509) were also collected from 17 water containers from 11 households. The DNA of the *Aedes* larvae and adults were screened for the presence of *Wolbachia* using the *wsp* and 16S markers, following optimized polymerase chain reaction (PCR) conditions, and sequenced. We also performed PCR using the mitochondrial DNA *cox1* marker and sequenced the *Wolbachia*-positive samples. Our results showed that 3 out of 359 (0.84%) adult *Ae. aegypti* and 12 out of 12 (100%) adult *Ae. albopictus* were *Wolbachia* positive, whereas all larvae tested negative for *Wolbachia* (0/509; 0%). The *wsp* marker revealed six *Wolbachia*-positive *Ae. albopictus*, whereas the 16S marker showed *Wolbachia* in

three *Ae. aegypti* and ten *Ae. albopictus.* Three *Wolbachia*-positive *Ae. albopictus* from one household were found to have a single haplotype for all *Wolbachia* (*wsp* and 16S) and mitochondrial *cox1* markers. This result can be used to infer that maternal transmission of *Wolbachia* in the household because siblings of a *Wolbachia*-infected mother mosquito share the *Wolbachia* and mitochondrial genomes. Our results suggest that the combined sequencing analyses of *Wolbachia* (*wsp* and 16S) and mitochondrial DNA can infer maternal transmission in field-collected mosquitoes.

Keywords

Wolbachia, dengue, *Aedes sp.,* haplotype, mitochondrial DNA

Introduction

Wolbachia is a maternally inherited endosymbiotic bacteria, infecting 40% of arthropod species and some filarial nematodes (Werren et al., 2008; Zug et al., 2012). Maternal transmission permits the rapid spread of *Wolbachia* in mosquito populations (Fraser et al., 2017). It is naturally present in medically important mosquito species, including *Aedes albopictus* (Albuquerque et al., 2011; Joanne et al., 2015; Kitrayapong et al., 2002) and *Aedes aegypti* (Balaji et al., 20119;Carvajal et al., 2019; Kulkarni et al., 2019; Teo et al., 2017) inhibiting the replication of arboviral pathogens (Iturbe-Ormaetxe et al., 2011). Natural populations of *Ae. aegypti* were considered to be negative for *Wolbachia*, but recent studies have reported both positive (Coon et al., 2016; Teo et al., 2017;Thongsripong et al., 2018; Hegde et al., 2018; Balaji et al., 2019; Kulkarni et al., 2019; Carvajal et al., 2019 and Bennett et al., 2019) and negative (Gloria-Soria et al., 2018 and Ross et al., 2019) results for *Wolbachia* in field-collected *Ae. aegypti* among different countries. Due to these varying results, further validation of natural infection of *Wolbachia* in field-collected *Ae. aegypti* is needed.

46 In the field, inference of maternal transmission is usually made by collecting adult
47 mosquitoes (Hoffman et al., 1998) or placing ovitraps (Schmidt et al., 2018) for *Wolbachia*
48 detection using polymerase chain reaction (PCR). In a study by Schimdt et al., 2018, ovitraps
49 were placed in field-released areas in Australia and three mosquito eggs per ovitrap were then
50 collected. Using the fluorescent real-time PCR/high resolution melt assay, 60 of 161 (37.27%)
51 *Ae. aegypti* were found to be *Wolbachia*-positive, indicating that full-siblings from most
52 ovitraps were able to sustain *Wolbachia* transmission in the field. The authors inferred
53 maternal transmission of *Wolbachia* because three eggs were collected from the same ovitrap
54 and, therefore, are assumed to be from the same mother. Notably, this method can only be
55 applied to ovitrap-based samples. In addition, they tested maternal transmission in Wolbachia-
56 released areas. Thus, in the present study, we used adult mosquitoes collected from the field
57 to infer maternal transmission of *Wolbachia*. If we need to see maternal transmission in
58 naturally occurring strains, this approach cannot be applied.

59 Since *Wolbachia* and mitochondrial genomes are maternally inherited, siblings of a
60 *Wolbachia*-infected mother mosquito will share these genomes, showing close association
61 leading to linkage disequilibrium among *Wolbachia*-infected mosquitoes. Maternal
62 transmission can be inferred through the analysis of both the *Wolbachia* and mitochondrial
63 genomes, indicating whether two or more *Wolbachia*-infected mosquitoes living in the same
64 or close localities (e.g., households) carried *Wolbachia* from the same mother. Yeap et al.
65 (2016) reported different mitochondrial DNA haplotypes between *Wolbachia*-transfected
66 mosquitoes from laboratory colonies and field-caught *Wolbachia*-negative mosquitoes. Based
67 on this result, they confirmed that the maternal transmission of *Wolbachia* occurs only in
68 mosquitoes with the same mitochondrial lineage as the transfected mosquitoes used for field
69 release. However, no study to date has assessed mosquito mitochondrial DNA haplotypes

70 along with *Wolbachia* sequences nor have any studies inferred maternal transmission of
71 naturally occurring *Wolbachia* strains from field-collected mosquitoes in areas without prior
72 release of *Wolbachia*-transfected mosquitoes.

73 The presence of natural *Wolbachia* strains in field-collected *Ae. aegypti* is important as
74 it may affect future *Wolbachia* mass release programs, thereby influencing the successful
75 invasion of *Wolbachia* strains from transfected mosquitoes into the natural population. Most
76 studies have found that naturally occurring *Wolbachia* in *Ae. aegypti* are strains that are
77 phylogenetically close to the wAlbA strain, which also infects *Ae. albopictus* (Balaji et al.,
78 2019;Carvajal et al., 2019; Kulkarni et al., 2019; Coon et al., 2016). *Ae. albopictus* is known to
79 be infected with two *Wolbachia* strains, known as wAlbA and wAlbB, which belong to the
80 supergroups A and B, respectively (Zhou et al., 1998). However, previous studies detecting
81 *Wolbachia* in field-collected *Ae. aegypti* did not test *Ae. albopictus*. To our knowledge, there
82 have been no previous studies that found *Wolbachia* supergroups A and B from *Ae. aegypti*
83 and *Ae. albopictus* samples collected from the same localities at the same time.

84 In this study, we detected *Wolbachia* in field-caught *Ae. albopictus* and *Ae. aegypti*
85 using *Wolbachia*-specific DNA markers (*wsp* and 16S). Combining the *Wolbachia* sequence
86 information with the mitochondrial DNA (*cox1*) sequence information, we inferred the
87 maternal transmission of *Wolbachia* from a common female ancestor. We tested the
88 hypothesis that *Wolbachia*-positive *Ae. albopictus* and/or *Ae. aegypti* mosquitoes collected
89 from same household may have the same haplotype in the mitochondrial DNA (*cox1*) and
90 *Wolbachia* markers (*wsp* and 16S), using adult samples collected from Manila City, Philippines.
91 We also identified and compared the *Wolbachia* strains and supergroups found in *Ae.*
92 *albopictus* and *Ae. aegypti*.

93 **Methods**

Study site, adult mosquitoes, larvae collection, and identification

We selected the study site in Manila City, the capital city of the Philippines. It is a highly populated and urbanized area that connects two major cities, Manila City and Quezon City. It consists of residential, commercial, and industrial infrastructure. We collected adults and larvae between June and September 2017, the rainy season, and the peak time for dengue cases.

Adults of 12 *Ae. albopictus* and 359 *Ae. Aegypti* were collected using commercially available mosquito light traps (Jocanima Corporation, Manila, Philippines) from 183 of 428 households that were randomly selected and surveyed. We set the mosquito trap for 48 hours inside or outside the surveyed households. The mosquito trap attracts the host-seeking and blood-fed mosquitoes to enter a capture net via the heat and CO_2 transmitted by a strong current from the ventilator (Balingit et al., 2020; Regilme et al., 2021). We also surveyed water containers in each household (*n* = 428) and found 17 containers with *Aedes* larvae (*n* = 509) outside the premises of 14 households. We classified the breeding containers into four general types following the World Health Organization guidelines (WHO, 2011) —water storage containers, such as drums, pails, and buckets, (n = 8); plant pots (n = 2); water fountain (n = 2); and others (namely, a discarded toilet bowl and a street canal; n = 2). The number of larval samples collected per water container ranged from 2 to 287 (mean = 31). The adults (*n* = 371) and larvae (*n* = 509) collected were morphologically identified as *Aedes sp.* using a stereomicroscope and the keys published by Rueda et al. (2004). The adult and larval samples were preserved in RNAlater (Ambion, Invitrogen, CA) solution to keep their RNA and DNA intact and stored at −20 before nucleic acid extraction.

Using microsatellite data from a recent study (Regilme et al., 2021) , we were also able to confirm the species of 359 adult mosquitoes as *Ae. aegypti.* We had already used the adult

118 DNA of 359 *Ae. aegypti* for population genetics study (Regilme et al., 2021) and used the
119 remaining DNA for this study for *Wolbachia* detection. The microsatellite primers were
120 species-specific for *Ae. aegypti* (Chambers et al., 2007; Slotman et al., 2007). The remaining 12
121 adult samples that did not show PCR amplification with the microsatellite primers were
122 confirmed to be *Ae. albopictus*.

123 **DNA extraction, molecular identification, PCR amplification, and sequencing**

124 We extracted DNA using Qiagen AllPrep DNA/RNA micro kit and Qiagen DNA Blood
125 and Tissue DNEasy Kits© (Qiagen, Hilden, Germany) in adult (*n* = 371) and larval (*n* = 509)
126 samples, respectively. DNA concentration and quality were measured using a NanoDrop 2000
127 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

128 *Wolbachia* was detected using two molecular markers: *wsp* (610 base pairs) using the
129 primer pairs *wsp* 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp* 691R (5′-
130 AAAAATTAAACGCTACTCCA-3′) (Zhou et al., 1998) and 16S specific for *Wolbachia* (850 base
131 pairs) with the primer pairs *WolbF* (5′-GAAGATAATGACGGTACTCAC-3′) and *Wspecr* (5′-
132 AGCTTC GAGTGAAACCAATTC-3′) (Simoes et al., 2011).

133 For both *wsp* and 16S gene amplification, we followed the protocol published in
134 (Carvajal et al., 2019), in a final volume of 10 µl with 1 µl of the genomic DNA. We used the
135 following components for the PCR reaction for both markers: 10x Ex Taq buffer, 25 mM $MgCl_2$,
136 2.5 mM dNTP, 10 µM forward and reverse primers, 10% dimethyl sulfoxide (Sigma-Aldrich, St.
137 Louis, MO, USA), and 5 units/µl of Takara Ex Taq™ (Takara Bio Inc., Shiga, Japan). The *wsp* PCR
138 amplification was as follows: initial denaturation at 95° for 3 minutes, denaturation at 95° for
139 1 minute, annealing at 55° for 1 minute, an extension at 72° for 1 minute for 40 cycles, and a
140 final extension at 72° for 3 minutes. The 16S amplification followed these conditions: initial

141 denaturation at 95□ for 2 minutes, denaturation at 95□ for 2 minutes, annealing at 60□ for 1
142 minute, an extension at 72□ for 1 minute for two cycles, another 35 cycles of denaturation at
143 95□ for 30 seconds, annealing at 60□ for 1 minute, an extension at 72□ for 45 seconds, and a
144 final extension at 72□ for 10 minutes. We included a positive control of a *Wolbachia*-positive
145 *Culex sp.* and negative control of water in each PCR run.

146 PCR products were analyzed in 1.5% agarose gel stained with Midori Green Advance
147 DNA stain at 100 V for 30 minutes. To validate the presence of *Wolbachia* in each sample, we
148 performed PCR amplification twice per marker. The criteria for a positive *Wolbachia* test were
149 based on two successful amplifications per molecular marker, *wsp,* and 16S.

150 We also amplified the *cox1* mitochondrial gene of *Wolbachia*-positive samples using
151 the primer pairs LCO-1490 (5□-GGTCAACAAATCATAAAGATATTGG-3□) and HCO1-2198 (5□-
152 AAACTTCAGGGTGACCAAAAAATCA-3□) (Folmer et al., 1994). We used the following PCR
153 amplification profile: initial denaturation at 95□ for 5 minutes, denaturation at 95□ for 30
154 seconds, annealing at 48□ for 45 seconds for 35 cycles, and a final extension at 72□ for 7
155 minutes. The amplified PCR products were purified using QIAquick (Qiagen, Hilden, Germany)
156 PCR Purification kits and sequenced by Eurofin Genomics Inc. Tokyo, Japan.

157 **Identification of *Wolbachia* strains, haplotypes, and phylogenetic analysis**

158 We assembled the forward and reverse sequences for each marker using the
159 CodonCode Aligner version 1.2.4 software (https://www.codoncode.com/aligner/). We aligned
160 the sequences using the online program MAFFT version 7 with the default settings
161 (https://mafft.cbrc.jp/alignment/software/). We checked all generated sequences for
162 similarities with reference sequences from GenBank (NCBI, 2016) using Basic Local Alignment
163 Search Tool–Nucleotide BLAST

164 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Finally, we checked the
165 sequence quality of the aligned sequences in Mesquite version 3.5 (Maddison et al., 2019) by
166 confirming the absence of stop codons.

167 All *wsp,* 16S, and *cox1 s*equences were separately analyzed using DNAsp version
168 6.12.03 (Rozas et al., 2017) to determine the number of haplotypes. We assessed the
169 relationship of the *Wolbachia* strains of our study with representative sequences from
170 different insect hosts by constructing a phylogenetic tree for the *wsp* and 16S sequences using
171 PhyML 3.1 (Guindon et al., 2003) using the default settings. We applied the GTR + G model for
172 *wsp* and the GTR + G + 1 model for 16S. The model per marker was selected using the SMART
173 model selection method (Lefort et al., 2017). We used *Brugia pahangi* (AY527207) and
174 *Rickettsia sp.* (U11021) as the outgroups for the *wsp* and 16S, respectively.

175 **Results**

176 **Detection of *Wolbachia* infection in adult and larval mosquitoes**

177 We screened all individual *Aedes sp.* larvae (*n* = 509) and adults (*n* = 371) for
178 *Wolbachia* using the *wsp* and 16S markers. Larval samples showed no evidence of *Wolbachia*
179 from either marker. However, *Wolbachia* was detected in 15 (4.04%) out of 371 *Aedes sp.*
180 adult mosquitoes based on the *wsp* and 16S markers. A total of three (0.84%) were *Wolbachia*
181 positive out of 359 *Ae. aegypti*, based on the *wsp* marker and 12 (100%) out of 12 *Ae.*
182 *albopictus* (Figure 1), based on the *wsp* and 16S markers. The *wsp* marker detected *Wolbachia*
183 in six *Ae. albopictus,* whereas the 16S marker detected it in three *Ae. aegypti* and ten *Ae.*
184 *albopictus*. Among these *Wolbachia*-infected *Ae. albopictus,* 4 (26.67%) out of 15 were positive
185 for both *wsp* and 16S markers. Confirmation of the species identification using the *cox1* marker

186 revealed that the 12 adult mosquitoes were *Ae. albopictus*, whereas the remaining three were
187 *Ae. aegypti* based on BLAST results with 100% identity match (Table 1).

188 The phylogenetic tree built using the *wsp* marker (Figure 2 A) revealed two major
189 clades: supergroup A and supergroup B (Zhou et al., 1998). Two *Ae. albopictus* were infected
190 with *Wolbachia* belonging to supergroup A and four with *Wolbachia* belonging to supergroup
191 B. The haplotype sequences from supergroup A were grouped with the *Wolbachia* type strain
192 A (wAlb A), identified in an *Ae. albopictus* host in the USA (Zhou et al., 1998). In contrast, the
193 sequences from supergroup B clustered with the *Wolbachia* type strains from *Ae. aegypti*
194 (*wAegB*) in the Philippines (Balaji et al., 2019; Carvajal et al., 2019) and *Ae. albopictus* (*wAlbB*)
195 in the USA (Zhou et al., 1998).

196 In the phylogenetic tree constructed using 16S sequences, 11 out of 13 sequences
197 (84.62%) found in *Ae. albopictus* (n = 10) and *Ae. aegypti* (n = 3) were clustered within
198 supergroup A (Figure 2 B). The remaining two sequences (15.38%) found in *Ae. albopictus* were
199 clustered together with the supergroups C, D, E, F, and J from insect hosts such as *Folsomia*
200 *candida, Mansonella azzardi,* and *Dirofilaria immitis.*

201 ***Wolbachia* and mitochondrial *cox1* haplotypes**

202 We identified six *cox1,* three *wsp,* and nine 16S haplotypes among the 15 *Wolbachia*-
203 positive sequences (Table 1). We found that three *Ae. albopictus* samples collected from one
204 household (NH149) were *Wolbachia* positive, based on the *wsp* and 16S markers (Table 1). An
205 identical *cox1* haplotype (sequence), C1, was observed among all three *Ae. albopictus*. The
206 same haplotypes of *wsp* (W2) and 16S (S6) also occurred among these three *Ae. albopictus*
207 (Table 2). Another household (NH56) had three *Wolbachia*-positive *Ae. albopictus* with the
208 same *cox1* haplotype (C1). Two *Ae. albopictus* (Individual codes 177 and 178) were *Wolbachia*

209 positive according to the *wsp* sequences, while two *Ae. albopictus* (Individual codes 95 and
210 177) were positive, according to the 16S sequence. We observed different haplotypes in each
211 *wsp* (W1 and W2) and 16S (S8 and S9) marker. Other than these two households, no other
212 households had more than one *Wolbachia*-positive mosquito.

213 **Discussion**

214 The presence of the same haplotype of *Wolbachia* (*wsp* and 16S) and mitochondrial
215 DNA (*cox1*) among the three *Wolbachia*-positive *Ae. albopictus* collected from household
216 NH149 may infer the maternal transmission of the *Wolbachia*.

217 We assumed that our approach eliminates the bias of laboratory-controlled conditions,
218 including temperature, light, and feeding times, during mosquito rearing. The maternal
219 transmission of *Wolbachia* in mosquitoes is usually confirmed by detecting *Wolbachia* in the
220 offspring of infected mothers (Balaji et al., 2019; Kulkarni et al., 2019). For example,
221 *Wolbachia*-microinjected *Ae. aegypti* embryos were reared into adults and then tested using
222 PCR for the presence of *Wolbachia* (McMeniman et al., 2009). Severe environmental
223 conditions such as high temperature causing heat stress to mosquitoes may decrease
224 *Wolbachia* density, thus reducing the ability of *Wolbachia* to persist in the mosquito
225 population. The results of a previous study (Ross et al., 2019) suggest that high temperature
226 affects *Wolbachia*. For example, when breeding containers are exposed to more sunlight than
227 shade, the density of *wMel* declined. Previous sterile insect studies failed to successfully
228 deploy *Wolbachia* because of the poor performance of the mosquitoes released in the field,
229 possibly because of the extreme environmental conditions differing from those of the
230 laboratory (Benedict et al., 2003; Helinski et al., 2013). Thus, it is important to verify *Wolbachia*
231 transmission in the field. In a previous study (da Silva et al., 2019), the establishment of
232 *Wolbachia* in Australia during and after mass release was determined by collecting mosquitoes

233 in the field and then detecting *Wolbachia* using loop-mediated isothermal amplification. The
234 researchers successfully confirmed maternal transmission because the field-collected
235 mosquitoes had wMel strains, which were considered absent from the natural populations
236 before mass release. However, the inference of maternal transmission of naturally occurring
237 *Wolbachia* strains in mosquito populations is not as simple as the case after mass release. It is
238 difficult to determine whether the strains detected in field-collected mosquitoes resulted from
239 transmission from the mother or the naturally occurring *Wolbachia* populations in the area.
240 Our approach, the combined analysis of *Wolbachia* and mitochondrial DNA sequences, can be
241 used to infer maternal transmission in field-collected mosquitoes.

242 One of the limitations of this study is that we observed an unexpected pattern found
243 in one household, NH56, that did not show the same haplotype of *Wolbachia wsp* and 16S
244 sequences in three *Wolbachia*-infected individuals. However, the same mitochondrial DNA
245 haplotype appeared in the three individuals. One of the possible reasons is that the resolution
246 of the *cox1* marker might not be fine enough to tell whether the mosquitoes with the same
247 *cox1* haplotype are from the same mother. Based on the number of *cox1* haplotypes [i.e., 4
248 (C1–C4)] found in the 12 *Wolbachia*-positive *Ae. albopictus*, we calculated a Poisson probability
249 of 0.149 for obtaining the same *cox1* haplotype among 3 randomly selected individuals. Our
250 study detected 6 *cox1* haplotypes from the 15 *Wolbachia*-positive individuals, and some
251 mosquitoes from the same or different households shared the same *cox1* haplotype, which
252 might imply that although they are not from the same household, they may have the same
253 maternal lineage. The mitochondrial genome reflects the long-term maternal lineage (Folmer
254 et al., 1994). Therefore, although the mothers of these three individuals were different, they
255 are considered to have a common ancestor in their maternal lineage, although how many
256 generations ago cannot be estimated. In future studies, we suggest increasing the sample size

257 to obtain more *Wolbachia*-positive mosquitoes from one household for a stochastically strong
258 validation of the maternal transmission through whole genome sequencing or double-digest
259 restriction site-associated DNA. The use of additional mitochondrial markers or whole genome
260 sequencing (e.g., double-digest restriction site-associated DNA) is also recommended for more
261 accurate verification.

262 Our results were negative for *Wolbachia* in all the collected *Aedes* larvae (n = 509).
263 One possible reason is that we found only 17 water containers during our field sampling. Since
264 *Wolbachia* is maternally transmitted, all of its offsprings would also be negative for *Wolbachia*
265 if the mother is negative for *Wolbachia*. More *Wolbachia* might be detectable in larval samples
266 if we increase the number of water containers than individuals per container. Although 428
267 households were surveyed in this study, only 17 water storage containers were found. Thus,
268 we suggest increasing the number of sampled water containers in future studies by setting up
269 ovitraps in households. We also recommend detecting *Wolbachia* in representative larval
270 samples from one water container.

271 Another possible reason for obtaining negative results for *Wolbachia* in larvae can be
272 linked to a lower *Wolbachia* density during the larval stage of mosquitoes. Previous studies
273 (Coon et al., 2016; Stevanovic et al., 2015) have found that the *Wolbachia* density is much
274 lower in the larval stage than in the adult stage. Wiwatanaratanabutr et al. (2009) discovered
275 that temperature and larval crowding may affect the relative *Wolbachia* densities in *Ae.*
276 *aelbopictus*. Stevanovic et al., (2015) found a lower *Wolbachia* density in *Drosophila*
277 *melanogaster* larvae than in adult females. We presume that this is due to the immature
278 organs of larva, such as the thoracic ganglia and muscles, ovaries, head, and salivary glands. In
279 the present study, we were unable to quantify the *Wolbachia* density in *Wolbachia*-positive

280 individuals. Thus, we recommend performing real-time quantitative PCR analysis alongside
281 conventional PCR in future studies.

282 We also found that the *Ae. albopictus* were infected with both supergroups A and B of
283 *Wolbachia* using the *Wolbachia* surface protein gene (*wsp*), widely used for *Wolbachia* strain
284 identification and phylogeny studies (Zhou et al., 1998). A previous study (Carvajal et al., 2019)
285 found that field-collected *Ae. aegypti* positive for *Wolbachia* from Manila, Philippines were
286 clustered into two supergroups: A and B. We found more *Wolbachia*-positive *Ae. albopictus* in
287 the *Wolbachia* supergroup B (4/6; 66.66%) than in supergroup A (2/6; 33.33%), as observed in
288 a previous study (Hu et al., 2020) (China; 631/693; 91.05%). Thus, our research results support
289 previous studies, and we observed two *Wolbachia* supergroups in *Ae. albopictus* collected
290 from the same area at the same time. However, the proportions of *Wolbachia* supergroups
291 might change in field conditions due to the influence of temperature and other abiotic factors.

292 We found a low prevalence of *Wolbachia* in adult *Ae. aegypti*, which is concordant
293 with the previous results of Kulkarni et al. (2016) obtained from field-collected *Ae. aegypti* in
294 Florida, USA and those of Carvajal et al.'s study (2019) conducted in Metro Manila, Philippines.
295 In contrast, a high (>50%) prevalence rate was observed in the studies of Teo et al. (2017)
296 conducted in Malaysia and Kulkarni et al. (2016) conducted in New Mexico, USA. The low
297 *Wolbachia* prevalence rate in *Ae. aegypti* may be due to the low density of the *Wolbachia*
298 endosymbiont in *Ae. aegypti* (Carvajal et al., 2019); this is supported by the previous
299 metabarcoding studies of Kulkarni et al.(2019), Coon et al. (2016), and Thongsripong et al.
300 (2018), which all reported a low number of sequence reads in the *Ae. aegypti* midgut,
301 indicating a low density of *Wolbachia*. Although our results are limited since we did not
302 quantitatively measure *Wolbachia* density, our 40-cycle PCR amplification method adapted
303 from Carvajal et al. (2019) could infer that *Wolbachia*-positive *Ae. aegypti* might be present in

304 Metro Manila, Philippines. Further *Wolbachia* detection studies are thus needed to affirm
305 natural *Wolbachia* infection in field *Ae. aegypti.*

306 We acknowledge the uncertainties of *Wolbachia*-positive *Ae. aegypti* due to the 16S
307 marker and the conventional PCR method, e.g., the false-positive rate. In light of this
308 information, we were careful to ascertain positive *Wolbachia* in adult *Ae. aegypti*. Thus, we
309 used the primers of Simoes et al. (2011), which are known to produce fewer false-positive
310 results and negative detections of *Wolbachia*. We also performed repeated PCR tests of our
311 *Wolbachia*-positive samples to ensure successful *Wolbachia* detection as defined in the
312 previous study by Carvajal et al. (2020). In addition to conventional PCR, tests such as
313 fluorescent *in situ* hybridization and whole genome sequencing can be used to provide strong
314 support for the presence of *Wolbachia* in natural *Ae. aegypti* populations. Despite these
315 limitations, our results provide additional support for *Wolbachia* detection in field-collected *Ae.*
316 *aegypti*.

317 **Conclusions**

318 An interesting approach to infer the maternal transmission of *Wolbachia* in field-
319 collected mosquitoes can be through the analysis of *Wolbachia wsp* and 16S sequences
320 combined with mitochondrial sequences. Since *Wolbachia* and mitochondria are both
321 maternally inherited, a non-random association of the mitochondrial genome with the
322 *Wolbachia* endosymbiont can be observed. The strategy of combining mitochondrial and
323 *Wolbachia* sequence information can infer the maternal transmission of *Wolbachia* in field-
324 collected *Ae. albopictus* and *Ae. aegypti.* In this study, we used mosquitoes naturally infected
325 with *Wolbachia* collected from the same households. We suggest that they might be from the
326 same maternal lineage (e.g. mother, grandmother etc.) due to the presence of the same
327 mitochondrial haplotype sequences in mosquitoes collected from the same household. This

may also imply that the other set of mosquitoes in this study with the same mitochondrial DNA haplotype but a different *Wolbachia* strain came from the same maternal lineage. Therefore, the results of *Wolbachia wsp* and 16S sequences combined with mitochondrial DNA sequences can support the use of genome sequencing to infer maternal transmission in field-collected mosquitoes. Using field-collected mosquitoes allows us to infer maternal transmission in the environment, a phenomenon that is possible to be overestimated under laboratory conditions. Our findings may provide additional support for the maternal transmission of *Wolbachia* in naturally infected mosquitoes in the field and its ability to invade and persist in the population.

List of Abbreviations

PCR: polymerase chain reaction

DNA: deoxyribonucleic acid

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article and its additional files. All newly generated sequences are available in the GenBank database under the Accession Numbers _____.

Competing interests

349 The authors declare that they have no competing interests.

350 **Funding**

351 This study was supported in part by the Japan Society for the Promotion of Science (JSPS)
352 Grant-in-Aid Fund for the Promotion of Joint International Research [Fostering Joint
353 International Research (B)] under grant number 19KK0107, JSPS Grant-in-Aid for Scientific
354 Research (B) under grant number 21H02206, the JSPS Core-to-Core Program B Asia-Africa
355 Science Platforms under grant number JPJSCCB20190008, and the Endowed Chair Program of
356 the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation.

357 **Author's contributions**

358 MAFR and KW conceptualized and designed the experiment. MAFR and TI collected and
359 identified the adult and larval samples for this study. MAFR and TI performed the *Wolbachia*
360 detection and all the molecular experiments for this study. MAFR, TI and KW performed the
361 data analysis. MAFR and KW wrote the manuscript. All authors read and approved the final
362 manuscript.

363 **Acknowledgments**

364 We are thankful to all the households that participated in our sampling collection of
365 mosquitoes and larvae and to the village officials of Sampaloc, Manila, for their assistance and
366 support. We appreciate the help of Johanna Beulah T. Sornillo of the Research Institute for
367 Tropical Medicine, Philippines, for her technical assistance in the household sampling design.
368 We want to thank Katherine Viacrusis for her service in the fieldwork and Mr. Micanaldo
369 Francisco for constructing the sampling map. Our deepest thanks to Dr. Thaddeus M. Carvajal,
370 Dr. Divina Amalin, Dr. Mary Jane Flores, Dr. Joeselle M. Serrana for their valuable suggestions
371 and technical assistance. The authors would like to thank Enago (www.enago.jp) for the

English language review. Maria Angenica F. Regilme is a recipient of the Japanese Government (Monbukagakusho) Scholarship from the Ministry of Education, Science, Sport, and Culture of Japan.

Reference

Albuquerque AL, Magalhães T, Ayres CF. High prevalence and lack of diversity of Wolbachia pipientis in *Aedes albopictus* populations from Northeast Brazil. Memórias do Instituto Oswaldo Cruz. 2011 Sep;106(6):773-6.

Balaji S, Jayachandran S, Prabagaran SR. Evidence for the natural occurrence of Wolbachia in Aedes aegypti mosquitoes. FEMS microbiology letters. 2019 Mar;366(6):fnz055.

Balingit JC, Carvajal TM, Saito-Obata M, Gamboa M, Nicolasora AD, Sy AK, Oshitani H, Watanabe K. Surveillance of dengue virus in individual Aedes aegypti mosquitoes collected concurrently with suspected human cases in Tarlac City, Philippines. Parasites & vectors. 2020 Dec;13(1):1-3.

Benedict MQ, Robinson AS. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends in parasitology. 2003 Aug 1;19(8):349-55.

Bennett KL, Gómez-Martínez C, Chin Y, Saltonstall K, McMillan WO, Rovira JR, Loaiza JR. Dynamics and diversity of bacteria associated with the disease vectors *Aedes aegypti* and *Aedes albopictus*. Scientific reports. 2019 Aug 21;9(1):1-2.

Carvajal TM, Hashimoto K, Harnandika RK, Amalin DM, Watanabe K. Detection of Wolbachia in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. Parasites & vectors. 2019 Dec;12(1):361.

393 Chambers EW, Meece JK, McGowan JA, Lovin DD, Hemme RR, Chadee DD, McAbee K, Brown
394 SE, Knudson DL, Severson DW. Microsatellite isolation and linkage group identification in the
395 yellow fever mosquito *Aedes aegypti*. Journal of Heredity. 2007 May 1;98(3):202-10.

396 Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential
397 for development but vary greatly between local habitats. Molecular ecology. 2016
398 Nov;25(22):5806-26.

399 da Silva Gonçalves D, Hooker DJ, Dong Y, Baran N, Kyrylos P, Iturbe-Ormaetxe I, Simmons CP,
400 O'Neill SL. Detecting wMel Wolbachia in field-collected *Aedes aegypti* mosquitoes using loop-
401 mediated isothermal amplification (LAMP). Parasites & vectors. 2019 Dec;12(1):1-5.

402 Fraser JE, De Bruyne JT, Iturbe-Ormaetxe I, Stepnell J, Burns RL, Flores HA, O'Neill SL. Novel
403 Wolbachia-transinfected *Aedes aegypti* mosquitoes possess diverse fitness and vector
404 competence phenotypes. PLoS pathogens. 2017 Dec 7;13(12):e1006751.

405 Folmer O. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from
406 diverse metazoan invertebrates. Mol Mar Biol Biotech. 1994;3:294-9.

407 Gloria-Soria A, Chiodo TG, Powell JR. Lack of evidence for natural Wolbachia infections in
408 *Aedes aegypti* (Diptera: Culicidae). Journal of medical entomology. 2018 Aug 29;55(5):1354-6.

409 Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by
410 maximum likelihood. Systematic biology. 2003 Oct 1;52(5):696-704.

411 Hegde S, Khanipov K, Albayrak L, Golovko G, Pimenova M, Saldaña MA, Rojas MM, Hornett EA,
412 Motl GC, Fredregill CL, Dennett JA. Microbiome interaction networks and community structure
413 from laboratory-reared and field-collected *Aedes aegypti*, *Aedes albopictus*, and *Culex*
414 *quinquefasciatus* mosquito vectors. Frontiers in microbiology. 2018 Sep 10;9:2160.

415 Helinski ME, Harrington LC. Considerations for male fitness in successful genetic vector control
416 programs. InEcology of parasite-vector interactions 2013 (pp. 221-244). Wageningen Academic
417 Publishers, Wageningen.
418 Hoffmann AA, Hercus M, Dagher H. Population dynamics of the Wolbachia infection causing
419 cytoplasmic incompatibility in Drosophila melanogaster. Genetics. 1998 Jan 1;148(1):221-31.
420 Hu Y, Xi Z, Liu X, Wang J, Guo Y, Ren D, Wu H, Wang X, Chen B, Liu Q. Identification and
421 molecular characterization of Wolbachia strains in natural populations of Aedes albopictus in
422 China. Parasites & vectors. 2020 Dec;13(1):1-4.
423 Iturbe‐Ormaetxe I, Walker T, O'Neill SL. Wolbachia and the biological control of mosquito‐
424 borne disease. EMBO reports. 2011 Jun;12(6):508-18.
425 Joanne S, Vythilingam I, Yugavathy N, Leong CS, Wong ML, AbuBakar S. Distribution and
426 dynamics of Wolbachia infection in Malaysian *Aedes albopictus*. Acta tropica. 2015 Aug
427 1;148:38-45.
428 Kitrayapong P, Baimai V, O'Neill SL. Field prevalence of Wolbachia in the mosquito vector
429 *Aedes albopictus*. The American journal of tropical medicine and hygiene. 2002 Jan
430 1;66(1):108-11.
431 Kulkarni A, Yu W, Jiang J, Sanchez C, Karna AK, Martinez KJ, Hanley KA, Buenemann M, Hansen
432 IA, Xue RD, Ettestad P. Wolbachia pipientis occurs in *Aedes aegypti* populations in New Mexico
433 and Florida, USA. Ecology and evolution. 2019 May;9(10):6148-56.
434 Lefort V, Longueville JE, Gascuel O. SMS: smart model selection in PhyML. Molecular biology
435 and evolution. 2017 Sep 1;34(9):2422-4.
436 Maddison, W. P. and D.R. Maddison. 2019. Mesquite: a modular system for evolutionary
437 analysis. Version 3.5 http://www.mesquiteproject.org

438 McMeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M, Wang YF, O'Neill SL. Stable introduction
439 of a life-shortening Wolbachia infection into the mosquito Aedes aegypti. Science. 2009 Jan
440 2;323(5910):141-4.
441 NCBI Resource Coordinators. Database resources of the National Center for Biotechnology
442 Information. Nucleic Acids Res. 2016;44(D1):D7-D19. doi:10.1093/nar/gkv1290
443 Regilme MA, Carvajal TM, Honnen AC, Amalin DM, Watanabe K. The influence of roads on the
444 fine-scale population genetic structure of the dengue vector *Aedes aegypti* (Linnaeus). PLoS
445 neglected tropical diseases. 2021 Feb 26;15(2):e0009139.
446 Ross PA, Ritchie SA, Axford JK, Hoffmann AA. Loss of cytoplasmic incompatibility in Wolbachia-
447 infected *Aedes aegypti* under field conditions. PLoS neglected tropical diseases. 2019 Apr
448 19;13(4):e0007357.
449 Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE,
450 Sánchez-Gracia A. DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular
451 biology and evolution. 2017 Dec 1;34(12):3299-302.
452 Rueda LM. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated
453 with dengue virus transmission. Walter Reed Army Inst Of Research Washington Dc
454 Department Of Entomology; 2004 Aug 3.
455 Schmidt TL, Filipović I, Hoffmann AA, Rašić G. Fine-scale landscape genomics helps explain the
456 slow spatial spread of Wolbachia through the Aedes aegypti population in Cairns, Australia.
457 Heredity. 2018 May;120(5):386-95.
458 Simoes PM, Mialdea G, Reiss D, Sagot MF, Charlat S. Wolbachia detection: an assessment of
459 standard PCR protocols. Molecular Ecology Resources. 2011 May;11(3):567-72.

460 Slotman MA, Kelly NB, Harrington LC, Kitthawee S, Jones JW, Scott TW, Caccone A, Powell JR.
461 Polymorphic microsatellite markers for studies of *Aedes aegypti* (Diptera: Culicidae), the vector
462 of dengue and yellow fever. Molecular Ecology Notes. 2007 Jan;7(1):168-71.
463 Stevanovic AL, Arnold PA, Johnson KN. Wolbachia-mediated antiviral protection in *Drosophila*
464 larvae and adults following oral infection. Applied and Environmental Microbiology. 2015 Dec
465 1;81(23):8215-23.
466 Teo CH, Lim P, Voon K, Mak JJ. Detection of dengue viruses and Wolbachia in *Aedes aegypti*
467 and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur, Malaysia. Trop Biomed.
468 2017 Sep 1;34:583-97.
469 Thongsripong P, Chandler JA, Green AB, Kittayapong P, Wilcox BA, Kapan DD, Bennett SN.
470 Mosquito vector-associated microbiota: Metabarcoding bacteria and eukaryotic symbionts
471 across habitat types in Thailand endemic for dengue and other arthropod-borne diseases.
472 Ecology and evolution. 2018 Jan;8(2):1352-68.
473 Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nature
474 Reviews Microbiology. 2008 Oct;6(10):741-51.
475 Wiwatanaratanabutr I. Geographic distribution of wolbachial infections in mosquitoes from
476 Thailand. Journal of invertebrate pathology. 2013 Nov 1;114(3):337-40.
477 World Health Organization. Operational guide for assessing the productivity of *Aedes aegypti*
478 breeding sites. Geneva: TDR. 2011.
479 Yeap HL, Rašić G, Endersby-Harshman NM, Lee SF, Arguni E, Le Nguyen H, Hoffmann AA.
480 Mitochondrial DNA variants help monitor the dynamics of Wolbachia invasion into host
481 populations. Heredity. 2016 Mar;116(3):265-76.

482 Zhou W, Rousset F, O'Neill S. Phylogeny and PCR–based classification of Wolbachia strains
483 using *wsp* gene sequences. Proceedings of the Royal Society of London. Series B: Biological
484 Sciences. 1998 Mar 22;265(1395):509-15.
485 Zug R, Hammerstein P. Still a host of hosts for Wolbachia: analysis of recent data suggests that
486 40% of terrestrial arthropod species are infected. PloS one. 2012 Jun 7;7(6):e38544.

487

488

489

490

491

492

493

494

495

496

497

498

499

500

501

Table 1. Summary of sampling data and *Wolbachia* detection results

	Total
Surveyed households	428
Households with adult Aedes mosquitoes	183
Households with larval Aedes	11
Collected Aedes mosquitoes	371
Aedes albopictus	12
Aedes aegypti	359
Collected Aedes larvae	509
Wolbachia positive	
Larva	0
Adult Ae. albopictus	12
Adult Ae. aegypti	3
Households with Wolbachia-positive mosquitoes	11

Table 2. A detailed summary of the results of the Wolbachia infection in selected Aedes mosquitoes using wsp and 16S markers

	Household	***Molecular species identification***	***Individual code***	***Haplotypes***			
				cox1	***wsp***		**16S** (n = 13)*
					Supergroup	***wsp*** (n = 6)*	
1	SH9	*Ae. albopictus*	40	C1			S7
2	SH27	*Ae. albopictus*	306	C5			S3
3	SH134	*Ae. aegypti*	384	C6			S1
4	SH214	*Ae. albopictus*	425	C1			S6
5	NH43	*Ae. albopictus*	172	C4			S3
6	NH56	*Ae. albopictus* (n=3)	095, 177, 178	C1	A (177) and B (178)	W1 (177) and W2 (178)	S8 (095) and S9 (177)
7	NH107	*Ae. albopictus*	218	C5			S4
8	NH130	*Ae. aegypti*	116	C2			S1
9	NH131	*Ae. albopictus*	240	C5	A	W3	S5

10	NH149	*Ae. albopictus* (n = 3)	258, 259, 260	C1	B (258, 259, 260)	W2 (258, 259, 260)	S6 (258, 259)
11	NH181	*Ae. aegypti*	121	C3			S2
	Total number of haplotypes			**6**		**3**	**9**

522

523 * total number of *Wolbachia*-positive mosquitoes

524

525

526

527

528

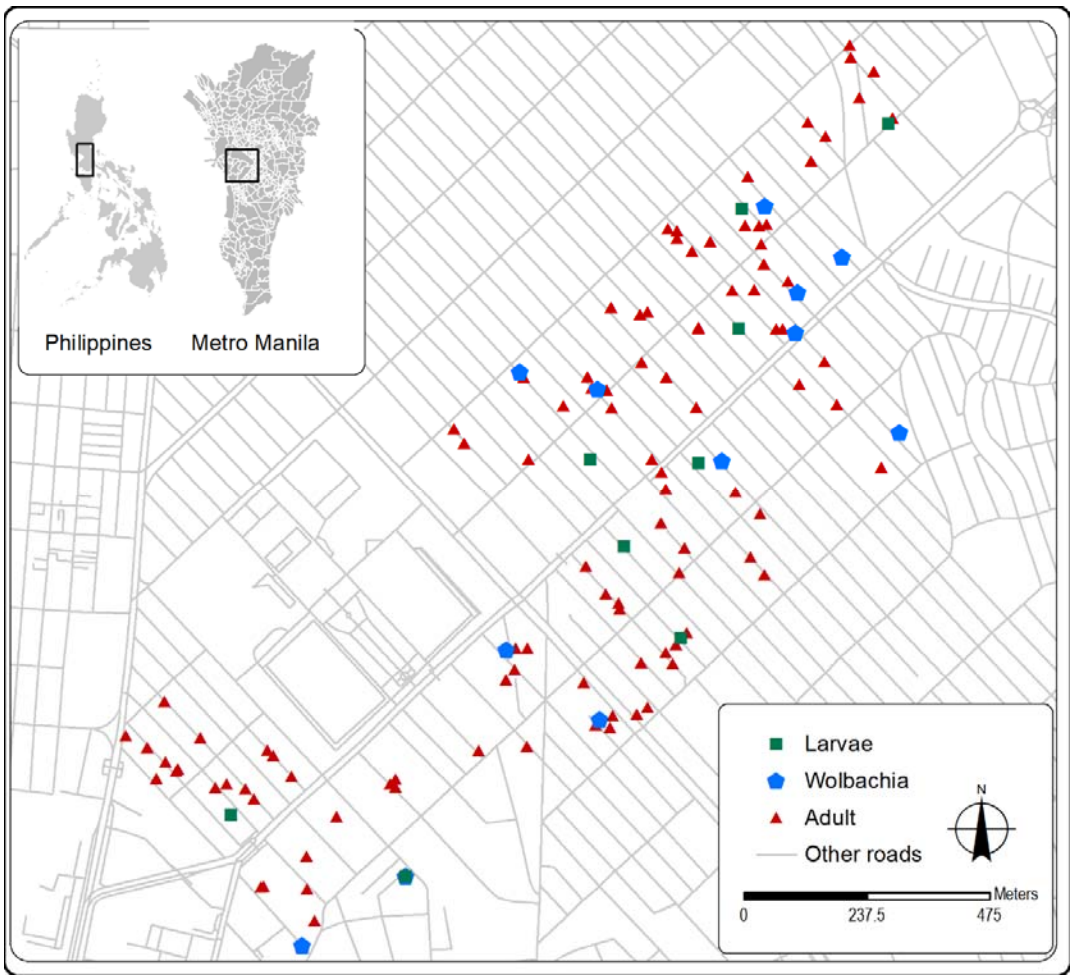
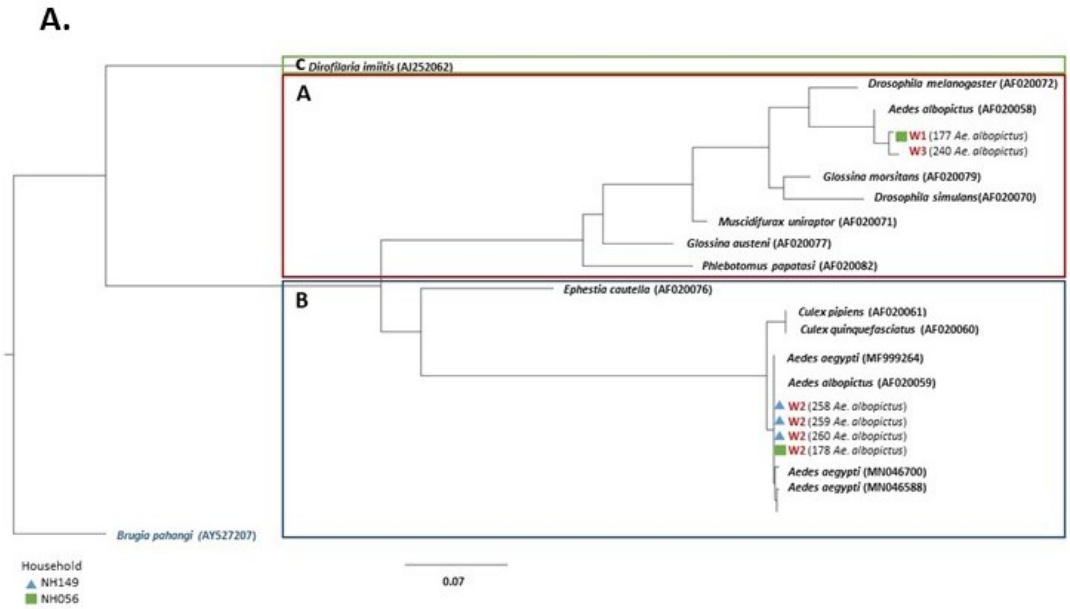
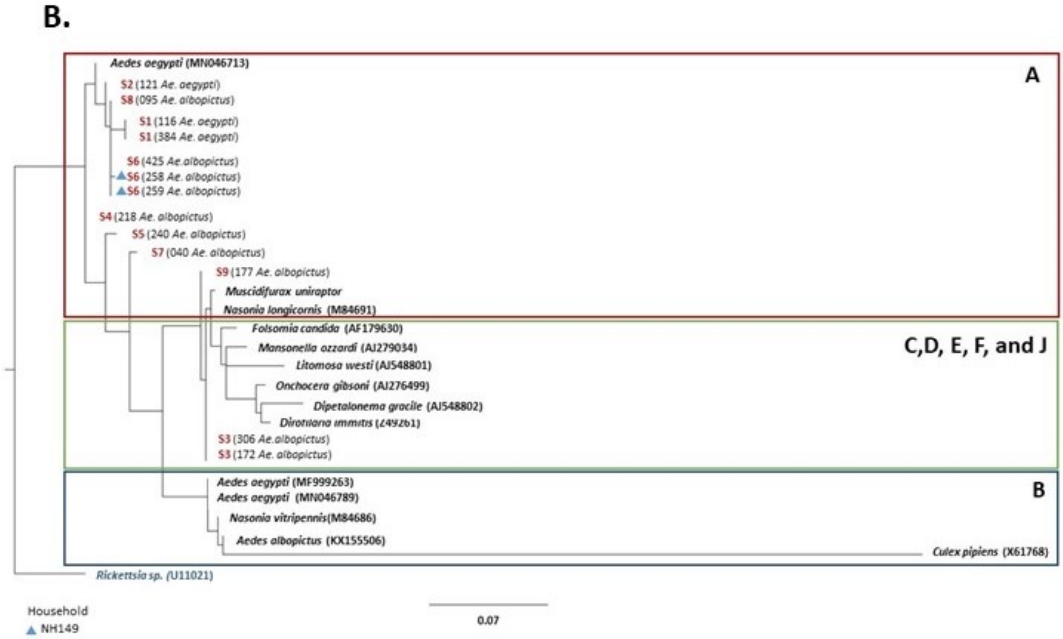


Figure 1. Spatial distribution of *Wolbachia*-positive households (n = 11) out of 428 households surveyed across a 2-kilometer road in highly urbanized area in Manila, Philippines.





536

537 **Figure 2.** The maximum likelihood phylogenetic tree of *Wolbachia*-infected urban mosquitoes

538 based on the **(A)** *WSP* sequences with reference sequences from supergroups A, B, and C and

539 an outgroup of *Brugia pahangi* **(B)** 16S sequences with reference sequences from supergroups

540 A, B, C, D, E, F, and J and an outgroup of *Rickettsia* sp.